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Studies on the interaction of α subunits of GTP-binding proteins with βγ dimers

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The interaction of several preparations of purified βγ dimers with two types of guanosine-nucleotide-binding-regulatory-(G)-protein α subunits, a recombinant bvα$_{i3}$, made in Sf9 *Spodoptera frugiperda* cells by the baculovirus (bv) expression system, and α$_s$, either purified from human erythrocyte G$_s$-type GTP-binding protein, and activated by NaF/AlCl$_3$, or unpurified as found in a natural membrane, were studied. The βγ dimers used were from bovine rod outer segments (ROS), bovine brain, human erythrocytes (hRBC) and human placenta and contained distinct ratios of β subunits that, upon electrophoresis, migrated as two bands with approximate M_r of 35000 and 36000, as well as distinct complements of at least two γ subunits each. When tested for their ability to recombine at submaximal concentrations with bvα$_{i3}$, ROS, brain, hRBC and placental βγ dimers exhibited apparent affinities that were the same within a factor of two. When bovine brain, placental and ROS βγ dimers were tested for their ability to promote deactivation of G$_s$, brain and placental βγ dimers were equipotent and at least 10-fold more potent than that of ROS βγ dimers; likewise, brain βγ and placental dimers were equipotent in inhibiting GTP-activated and GTP-plus-isoproterenol-activated adenylyl cyclase, while ROS βγ dimers were less potent when assayed at the same concentration. The possibility that different α subunits may distinguish subsets of βγ dimers from a single cell was investigated by analyzing the βγ composition of three G proteins, G$_s$, G$_{i2}$ and G$_{i3}$, purified to near homogeneity from a single cell type, the human erythrocyte. No evidence for an α-subunit-specific difference in βγ composition was found. These findings suggests that, in most cells, α subunits interact indistinctly with a common pool of βγ dimers. However, since at least one βγ preparation (ROS) showed unique behavior, it is clear that there may be mechanisms by which some combinations of βγ dimers may exhibit selectivity for the α subunits they interact with.

One of the characteristics of the trimeric signal transducing heterotrimetric GTP-binding proteins of αβγ subunit composition (G proteins) is that they can be activated by GTP analogs such as guanosine 5′-[γ-thio]triphosphate (GTP[γS]) to give two reaction products: a complex of the guanine nucleotide with the α subunit and a free βγ dimer. Although not yet proven unequivocally, the dissociation reaction is believed to occur also in membranes containing GTP, under the catalytic effect of ligand-activated receptors [1, 2]. It has been speculated [2] and proposed [3, 4] that both α subunits and βγ dimers may regulate effector functions under physiologic conditions. Such a role has indeed been firmly established for α subunits, which act at picomolar concentrations in several systems (summarized in [5]). For βγ dimers, which act in *in-vitro* assays at nanomolar concentrations, such a role has been debated [6–8]. Reasons for speculating that the βγ dimers participate in information transfer include the findings that βγ dimers mimic some aspects of inhibition of adenylyl cyclase [2], the finding that, in budding-yeast cells, a βγ dimer and not an α subunit mediates the effects of a pheromone [9–11], the recent discovery of the existence of molecular forms of adenylyl cyclase that are either stimulated or inhibited by high concentrations of βγ dimers [12, 4] and the realization that βγ dimers are molecularly quite diverse. In fact, while 16 genes encoding 17 α-subunit-gene products have been identified [13], it is possible to postulate a similar number of complexes also for βγ dimers, based on the existence of four highly similar β-subunit genes, a minimum of five γ-subunit genes [14] and perhaps several more as visualized by immunoreactivity [15, 16] and differential migration upon urea/SDS/PAGE.

In contrast, studies on reassociation of the α subunit of transducin with βγ dimers of different origins (and composition) had indicated that βγ subunits are functionally exchangeable [17–20]. This had led to the proposal that, in any given cell, the various βγ dimers may constitute a common pool, of which its members would be shared equally among the different α subunits expressed in that cell [6]. This would

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Abbreviations. bv, baculovirus; G protein, a class of heterotrimeric GTP-binding proteins of αbg subunit composition; G$_x$, G protein with αβγsubunit; GDP[βS], guanosine 5′-[β-thio]diphosphate; GTP[γS], guanosine 5′-[γ-thio]triphosphate; PTX, pertussis toxin; ROS, rod outer segment.

Enzymes. Adenylate kinase: ATP:ATP phosphotransferase (EC 2.7.4.3); adenylyl cyclase: ATP:pyrophosphate lyase (cyclizing) (EC 4.6.1.1); creatine phosphokinase: ATP:creatine *N*-phosphotransferase (EC 2.7.3.2); NAD-pyrophosphohydrolase: ATP:NMN adenylyltransferase (EC 2.7.7.1).

make it unlikely that portions of the holo-G protein other than the α subunit participate in the transfer of information from receptor to effector.

One approach to the question as to whether βγ dimers may be mediators of receptor signals, other than to test their effects on response systems directly, is to test for specificity in their interactions with α subunits. In this case, one would predict that, if βγ dimers are mediators of specific effects of G proteins, α subunits should have distinguishing affinities for one or another of the different types of βγ dimers and that the βγ dimer complement associated with a given α subunit should differ from one G protein to another.

We report below that, upon testing for the interaction of βγ dimers from four different sources [erythrocytes, retinal rod cell segments (ROS), placenta and brain], with several types of α subunits, we could not detect any significant differences in behavior among three of these βγ subunits (erythrocyte, brain and placental βγ). However, the fourth βγ (ROS) differed in its behavior from the others, as seen in two G_s-related tests (G_x, G protein with α_x composition), but not in a third test (trimer formation as seen by stimulation of ADP-ribosylation). The implications of these findings are discussed.

MATERIALS AND METHODS

Materials

Wild-type baculovirus (*Autographa californica* nuclear polyhidrosis virus), the expression plasmid pVL-941 and *Spodoptera frugiperda* Sf9 cells were a kind gift from Dr. Max Summers (Texas A & M University, College Station, TX). Cells to be infected with recombinant baculovirus, bearing the human α_{i3} cDNA, were kept in logarithmic growth phase in spinner flasks agitated at 60 cycles/min at 27°C, in Grace's medium supplemented with 0.33% lactalbumin hydrolysate, 0.33% yeastolate, 0.035% $NaHCO_3$, pH adjusted to 6.2 with NaOH, 1% (by vol.) of penicillin/streptomycin solution and 10% fetal bovine serum. Grace's medium, lactalbumin hydrolysate and penicillin/streptomycin solution were from Gibco. SeaKem agarose was from FMC Laboratories. Bovine serum albumin (Cohn fraction V), ovalbumin (egg albumin), carbachol, Lubrol-PX, Nonidet P-40, creatine phosphate, creatine phosphokinase and adenylate kinase were from Sigma Chemical Company. Prior to use, Lubrol PX was purified by ion exchange chromatography as described [21]. Fetal bovine serum was from Hazleton; TC yeastolate was from Difco Laboratorie. Nytran membranes were from Schleicher & Schuell. Chromatography media were DEAE-Sephacel and prepacked Mono-Q from Pharmacia. Polyacrylamide-gel electrophoresis supplies were from BioRad. DEAE-Toyopearl (TSK-gel Toyopearl DEAE 650 M) was from Supelco. Guanosine nucleotides, adenyl-5′yl imidodiphosphate, nicotinamide mononucleotide and NAD-pyrophosphorylase were from Boehringer Mannheim. Carrier-free ^{125}I was purchased from IsoTex. [α-^{32}P]ATP, prepared by the method of Walseth and Johnson [22], and [^{32}P]NAD^+, synthesized according to Cassel and Pfeuffer [23], were supplied by the Molecular Endocrinology Core Laboratory of the Baylor College of Medicine Diabetes and Endocrinology Research Center. All other reagents were of the highest purity commercially available and used without further purification.

Polyacrylamide-gel electrophoresis

Proteins were analyzed by SDS/PAGE according to Laemmli [24], utilizing as separating gels either a 10% polyacrylamide gel made in Laemmli's separation buffer (normal SDS/PAGE), a 9% polyacrylamide gel slab polymerized in a 4–8 M urea gradient dissolved in Laemmli's separation buffer (urea gradient/SDS/PAGE as described in Scherer et al. [25] and Codina et al. [26]) or a discontinuous-gel slab in which the upper half was 12.5% polyacrylamide polymerized in Laemmli's separation buffer without urea and the bottom half was a double gradient of 4–8 M urea and 12.5%–25% polyacrylamide gradient polymerized in Laemmli's separation buffer (discontinuous-urea-gradient and polyacrylamide-gradient electrophoresis or DUPAGE as described in Hildebrandt et al.[27]). For construction of urea gradients, the Laemmli separation buffer ingredients were dissolved in freshly prepared 9 M urea immediately after deionizing by passing up to 500 ml three times over an initially dry 50-ml column of 20–50-μm-mesh BioRad AG 501-X8(D) resin. Urea gradient/SDS/PAGE was used to maximize the separation of different forms of α and β subunits. Urea gradient/SDS/PAGE was used to maximize separation of different forms of γ subunits. Gels were stained either with Coomassie blue [21] or with Coomassie blue stained followed by silver staining to yield different colors [28]. For silver staining, the gels were destained for 24 h in 50% methanol and soaked another 12 h in 10% acetic acid/50% methanol.

Pertussis-toxin-catalyzed ADP-ribosylations

Prior to use, pertussis toxin (PTX) was activated by incubation at 250 μg/ml for 30 min at 32°C in the presence of 50 mM dithiotreitol and 1 mM adenyl-5′yl imidodiphosphate. This was then diluted 10-fold in 10 mM Tris/HCl, pH 8.0, and 0.025% bovine serum albumin and kept on ice until used. The ADP-ribosylation reactions contained 2 vol. 300 μM guanosine 5′-[β-thio]diphosphate (GDP[βS]), 30 mM thymidine, 3 mM EDTA, 3 mM ATP, 6 mM dithiotreitol, 0.5% Lubrol PX and 45 mM Tris/HCl, pH 8.0, 1 vol. diluted toxin, 1 vol. 1 μM [^{32}P]NAD^+ ($2-5\times10^6$ cpm) and 2 vol. additions containing up to 50 nM native G proteins, the indicated concentrations of βγ dimers, and up to 50 nM recombinant α_{i3} subunits in the absence or the presence of the indicated concentrations of βγ dimers, in 10 mM Hepes (sodium salt), pH 8.0, 1 mM EDTA, 20 mM 2-mercaptoethanol, 30% ethylene glycol, 0.1% Lubrol PX and 0.1% bovine serum albumin. Final volumes varied over 10–60 μl. Incubations were for 30 min at 32°C or 30–36 h at 4°C and the reactions were terminated by addition of 2–3 times the incubation volume of 2× Laemmli's sample buffer containing 4 mM unlabeled NAD. The ADP-ribosylated proteins were then subjected directly to SDS/PAGE or urea gradient/SDS/PAGE. The slabs were stained by Coomassie blue, to monitor efficiency of sample (bovine serum albumin) transfer onto the gels, and autoradiographed to monitor [^{32}P]ADP-ribosylation.

Immunoblots

Proteins were electrophoresed and transferred overnight at 25–30 mV at room temperature from unstained gel slabs onto nitrocellulose sheets, wetted with 25 mM Tris base, 190 mM glycine and 20% methanol using a BioRad Transblot apparatus. The sheets were then blocked for 3 h at room temperature by agitating in buffer A (buffer A, 150 mM NaCl, 0.1% NaN_3, 50 mM Tris/HCl, pH 8.5) plus 3% ovalbumin (cleared by centrifugation at $30000\times g$ for 15 min) and exposed to first antibody diluted in fresh buffer A plus 3% ovalbumin. The sheets were then subjected to 10-min washes

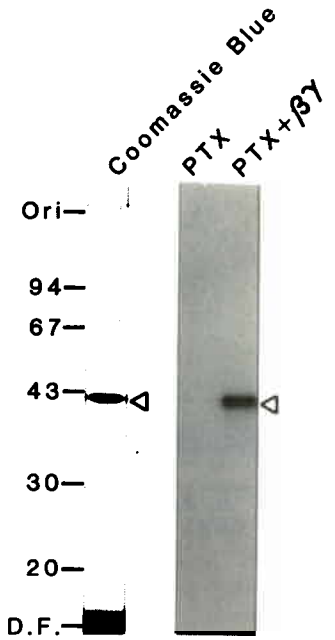

Fig. 1. Coomassie-blue stained and $\beta\gamma$ dimer-dependent ADP-ribosylation of purified bvα_{i3}. Left, SDS/PAGE analysis of 2 μg bvα_{i3} used in these studies (Coomassie-blue stain). Right, SDS/PAGE analysis of [^{32}P]ADP-ribosylation products obtained upon ADP-ribosylating 0.5 ng bvα_{i3} (reaction volume 15 μl) in the absence and presence of a 10-fold molar excess of brain $\beta\gamma$ (autoradiograph). Stds, M_r of 94000, 67000, 43000 and 21000 as described in [21]; D. F., dye front; Ori, origin.

at room temperature with buffer A, buffer A plus 0.1% Nonidet P-40 and twice with buffer A, blocked again with buffer A plus 3% ovalbumin (1 – 3 h), and added to buffer A plus 3% ovalbumin containing 1×10^6 cpm of ^{125}I-labeled second antibody, usually goat anti-(rabbit IgG) serum, for 3 – 15 h. After washing as before, the sheets were blotted to dryness, mounted onto Whatman number 1 paper and analyzed by autoradiography.

Rabbit anti-(Gα peptide) IgG were prepared against keyhole limpet hemocyanin-coupled cysteinyl peptides according to Green et al. [29]. Two such antisera, AI2 (anti-α_{i2}, directed against α_{i2}-(112 – 126) of the human α_{i2} sequence [30]) and AI3 (anti-α_{i3}, directed against cysteinyl-α_{i3}-(111 – 124) of the human α_{i3} sequence [31, 32]) were used at dilutions of 1:100 to 1:200, to identify the human G_i proteins as G_{i2} and G_{i3}. Iodination of the second antibody (5 μg) in 30 μl 600 mM potassium phosphate, pH 7.0, was carried out by addition of 2 mCi carrier-free ^{125}I solution in 2 – 5 μl, and 10 μl fresh 5 mg/ml solution of chloramine T. The reaction was stopped after 1 min at room temperature with 100 μl fresh 8.5 mg/ml of sodium metabisulfite. The labeled IgG was separated from the iodination reagents by molecular exclusion chromatography over a prepacked Pharmacia PD-10 column, equilibrated and developed with buffer A plus 0.1% ovalbumin.

Recombinant baculoviral α_{i3} subunits

Recombinant baculovirus (bv) was purified to greater than 90% purity (Fig. 1) from lysates of Sf9 cells infected with recombinant baculovirus bearing the open reading frame encoding the human α_{i3} (*NcoI* – *AvaII* fragment of α_{i3} in M13, described in [46] in place of the open reading frame of the polyhedrin gene, following procedures outlined in the text and to be described in detail elsewhere (Graf, R., Codina, J. & Birnbaumer, L., unpublished results). Recombinant baculovirus was obtained following procedures of Summers and collaborators [33, 34].

Purification of G_{i2} and G_{i3} from human erythrocyte membranes

Human erythrocyte G proteins were extracted from erythrocyte membranes (55 g protein) and subjected to the first-three chromatography steps (DEAE-Sephacel, AcA-34 Ultrogel and Heptylamine-Sepharose) as described for pool B of the AcA-34 eluate, which contains approximately 65% of the PTX substrates [21, 35]. The fractions were assayed for G_s (*cyc*$^-$ reconstituting activity) and for the presence of PTX substrates as seen by [^{32}P]ADP-ribosylation followed by urea gradient/SDS/PAGE and autoradiography. The latter shows two major PTX-labeled bands denoted as 'upper' and 'lower', which correspond to the ADP-ribosylated forms of α_{i3} and α_{i2}, respectively. Resolution of G_{i2} from G_{i3}, as well as separation from other proteins, was accomplished by sequential chromatography, first over a second heptylamine-Sepharose column, then over two or three DEAE-Toyopearl columns as shown in Fig. 2. G_s was purified from the AcA Ultrogel pool A, taking care to pool the eluates so as to minimize contamination with PTX substrates. A total of four pools of G proteins were obtained, with the following protein yields: 81 μg G_s (Fig. 3, lane S); 360 μg G_{i2} (Fig. 3, lane A); 414 μg G_{i3} (Fig. 3, lane B); a mixture of 450 μg G_s plus 220 μg G_{i2} (Fig. 3, lane C). The G_s, G_{i2} and G_{i3} proteins prepared in this way were at least 90% pure in terms of unrelated contaminants and contained less than 5% cross contaminating G proteins as assessed by PTX labeling and *cyc*$^-$ reconstitution assays (not shown).

'Upper' and 'lower' PTX substrates were identified as α_{i3} and α_{i2}, respectively, by immunoblotting with peptide-specific antisera AI2-1 and AI3-2. Antiserum AI2 reacted with the α subunit of the 'lower' G protein and not with that of the 'upper'; the converse was found with antiserum AI3 (not shown). Limited amino acid sequence analysis of *Staphylococcus aureus* V8-protease-digested peptide fragments derived from a sample formed of 90% 'upper'/10% 'lower' provided independent confirmation for the identity of 'upper' as α_{i3} [36].

Purification and assay of human erythrocyte G_s

G_s was purified free of PTX substrates and assayed by its ability to reconstitute GTP[γS]-stimulated, isoproterenol and GTP-stimulated and sodium-aluminum-fluoride-stimulated adenylyl cyclase activity in *cyc*$^-$ S49 cell membranes as described [21, 35].

$\beta\gamma$ dimers

Human erythrocyte $\beta\gamma$ dimers (Figs 4 and 5) were prepared as described [21, 35] and were frozen at −70 °C at 410 μg/ml (10 μM) in 200 mM NaCl, 20 mM 2-mercaptoethanol, 1 mM EDTA, 10 mM Hepes (Na salt), pH 8.0, 30% ethylene glycol and 0.1% Lubrol-PX.

Human placenta $\beta\gamma$ dimers (Figs 4 and 5) were purified [16] and kept frozen at −70 °C at a concentration of 317 μg/ml (7.78 μM) in 50 mM Hepes (Na salt), pH 8.0, and 0.1% Lubrol-PX.

Bovine brain $\beta\gamma$ dimers (Figs 4 and 5) were obtained as a side fraction upon purification of bovine brain G_o [36] and

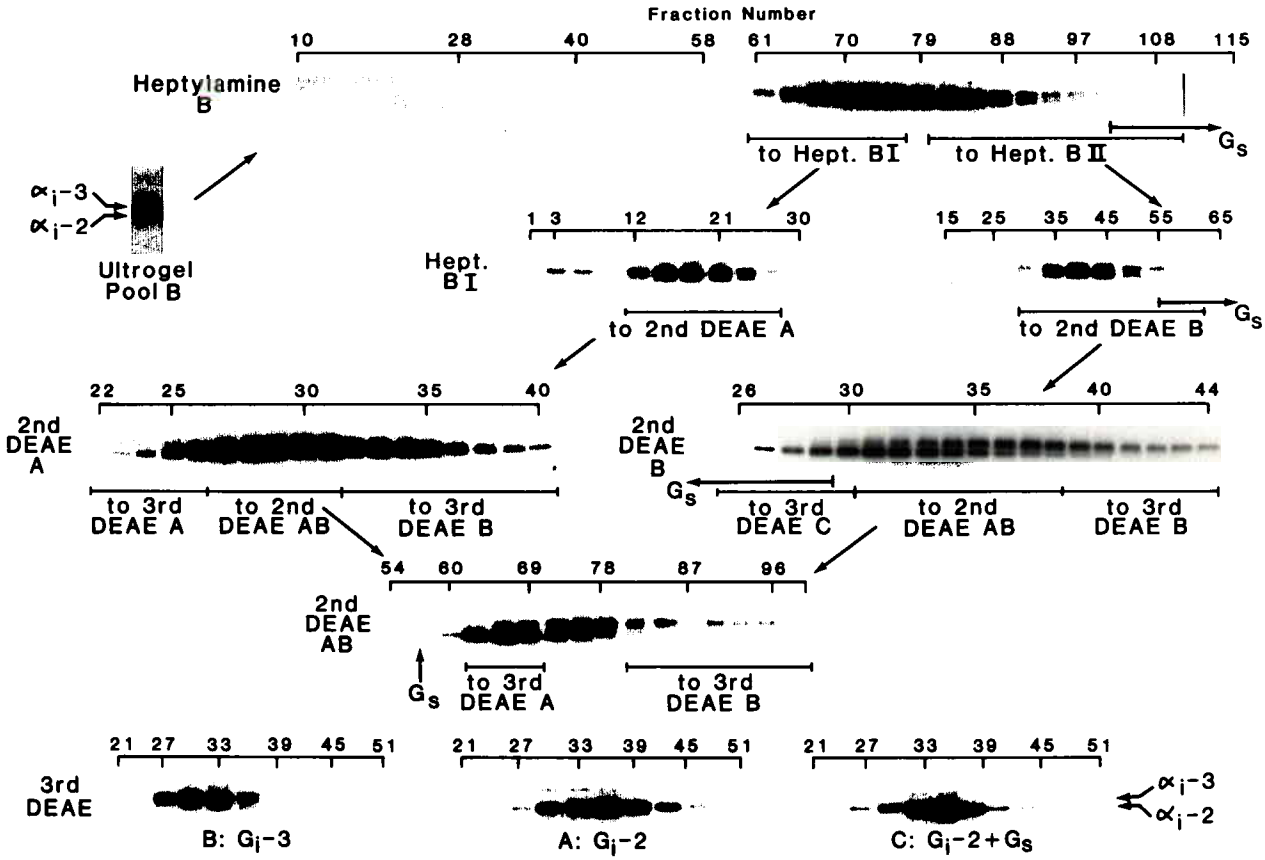

Fig. 2. Progress of the purification of human erythrocyte G_{i2} and G_{i3}. The figure shows the areas of autoradiograms of urea gradient/SDS/PAGE gels that correspond to α subunits of PTX-sensitive G proteins (M_r 35–45000). Pool B (230 ml) from an Ultrogel AcA 34 column was chromatographed over heptylamine-Sepharose [variant B: 2.5 cm diameter, 100 ml bed volume; 600-ml reciprocal 0.4–2% cholate/200–0 mM NaCl gradient in 20 mM 2-mercaptoethanol, 1 mM EDTA, 10 mM Hepes (Na salt, pH 8.0, 30% ethylene glycol; 6-ml fractions]. As shown, the 'upper' (leading) PTX substrate was partially separated from the 'lower' (trailing) PTX substrate, the last part of which in turn overlapped with G_s. Fractions were pooled as shown and rechromatographed over heptylamine-Sepharose (heptylamine B-I and B-II chromatographies; columns of 0.9-cm diameter, 10-ml bed volume; 600-ml reciprocal gradient as above, 6-ml fractions). The fractions with PTX substrates from these two steps were then subjected to separate second DEAE chromatographies over DEAE-Toyopearl (0.9-cm diameter, 10-ml bed volume; 600-ml 0–4% cholate gradient in buffer B plus 0.5% Lubrol-PX; 6-ml fractions). This resulted in a further partial separation of 'lower' (now leading) from 'upper' (now trailing) PTX substrate, with the difference that while the leading 'lower' PTX substrate eluting from the second DEAE-A was free of G_s, that eluting from the second DEAE-B contained an about equimolar amount of G_s. The eluates from each of the second DEAE columns were combined to give three pools of which the two center pools, (approximately 1:1 'upper' and 'lower' PTX substrates) were combined, diluted and re-chromatographed over DEAE-Toyopearl (second DEAE-AB; 0.9-cm diameter, 50-ml bed volume; 600-ml 0–4% cholate gradient as above; 6-ml fractions). The fractions with the leading PTX substrate ('lower') from the second DEAE-AB were combined with those from the second DEAE-A, the fractions with the trailing 'upper' PTX substrate were combined with those from both the second DEAE-A and DEAE-B. These and the pool of leading 'lower' PTX substrate plus contaminating G_s from the second DEAE-B pools were then subjected to a third DEAE chromatography (columns designated A, B and C as shown on the figure; 0.9-cm diameter; 10-ml bed volume; 600-ml 0–300 mM NaCl gradient in buffer B plus 0.5% Lubrol-PX; 6-ml fractions). 'Upper' PTX substrate (G_{i3}) elutes from the third DEAE column slightly earlier than the 'lower' PTX substrate (G_{i2}), which in turn essentially co-chromatographs with G_s. Fractions from the third DEAE A, B and C columns were pooled to give pools A (G_{i3}, Fig. 3, lane A), B (G_{i2}, Fig. 3, lane B) and C (G_s plus G_{i2}, Fig. 3, lane C). Flow rates were 1.0–1.1 ml. All other conditions and materials were as described in [34]. PTX labeling and urea gradient/SDS/PAGE were as described in Materials and Methods. Hept, heptylamine-Sepharose [21].

were kept frozen at −70°C at 355 μg/ml (8.66 μM) in the same buffer as human erythrocyte βγ.

Bovine ROS βγ dimers were prepared from rod transducin [37] and kept frozen at −70°C at 1.400 mg/ml (34 nM) in 10 mM Mops, 2 mM dithiotreitol, 200 mM NaCl, 2 mM $MgCl_2$ and 40% glycerol.

RESULTS

Assessments of interactions between βγ dimers and α subunits

The present experiments were designed to test the properties of βγ dimers as assessed by their ability to interact with α subunits. Three sets of experiments were performed. In one, we studied the βγ-dimer distribution, as assessed by β_{35}/β_{36} ratios, among three naturally occurring G proteins, G_s, G_{i2} and G_{i3}, purified to apparent homogeneity from a homogenous cell population, in this case human erythrocyte. In the second approach, we tested the ability of βγ dimers to promote ADP-ribosylation of an α subunit, in this case recombinant α_{i3} synthesized by Sf9 insect cells infected with a baculovirus in which we had inserted the α_{i3} cDNA downstream of the polyhedrin promoter. This approach is based on the fact tha ADP-ribosylation of any PTX-sensitive G-protein α subunit can be promoted by any βγ dimer, regardless of its origin. In the third approach, we compared the ability of three of the βγ

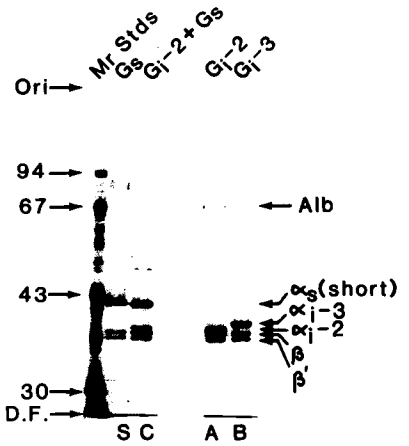

Fig. 3. Urea gradient/SDS/PAGE analysis of fractions from the purification shown in Fig. 2. Photographs of Coomassie blue stained gels are shown. Lanes S, A, B and C, correspond to aliquots of proteins obtained, respectively, from purifying G_s activity from the AcA 34 Ultrogel pool A, G_{i2}, G_{i3} and a mixture of G_s plus G_{i2} from the AcA Ultrogel pool B [35]. Note the presence of two β subunits in each of the proteins. γ subunits (M_r = 5000 – 8000) are not resolved on these gels and migrate with the dye front. Lanes S, C, A, and B received 250, 350, 250 and 500 ng protein, respectively. Ori, origin; D. F., dye front; Alb, albumin.

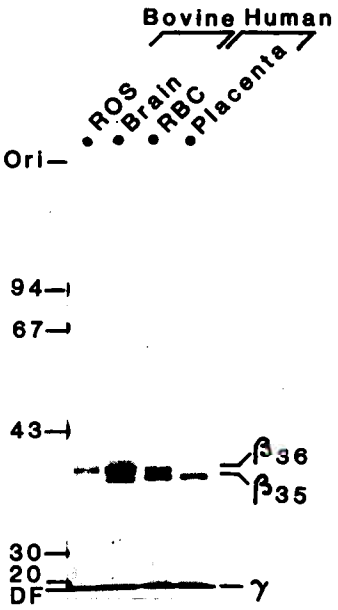

Fig. 4. Analysis of the distribution of β subunits with migration of β_{35} and β_{36} in $\beta\gamma$ dimers used in these studies. $\beta\gamma$ dimers were subjected to urea gradient/SDS/PAGE, the gel slab was stained with Coomassie blue and photographed. Protein samples were 312 ng bovine ROS $\beta\gamma$; 1200 ng bovine brain $\beta\gamma$, 687 ng human erythrocyte $\beta\gamma$ (RBC) and 375 ng human placental $\beta\gamma$. γ subunits were not resolved from the dye front (D. F.). Ori, origin; Alb, albumin; Stds, standards. The M_r standards are shown in the left-hand lane.

preparations to interact *in vitro* with G_s, as seen by their ability to promote deactivation of fluoride-activated human erythrocyte G_s and their ability to inhibit membrane-bound adenylyl cyclase.

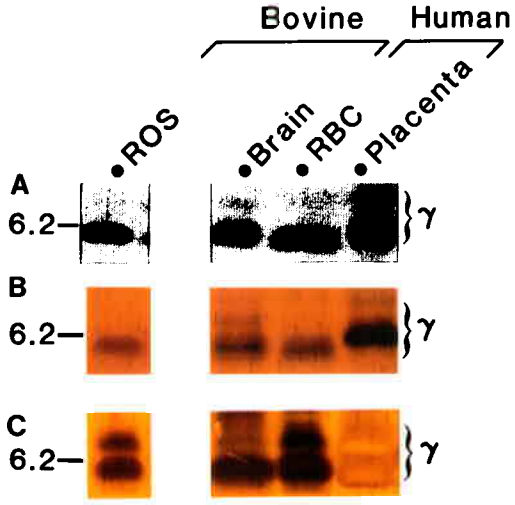

Fig. 5. Analysis of γ peptides in the $\beta\gamma$ dimers used in these studies. $\beta\gamma$ dimers were subjected to discontinuous-urea-gradient and polyacrylamide gradient gel electrophoresis in the presence of SDS as described in Materials and Methods and the gel slabs were stained first with Coomassie blue and photographed, then, after destaining overnight in methanol, with a color-developing silver stain [28] and photographed again. The figure shows only the regions of the gels in which proteins are that migrate with apparent molecular masses of 2000 – 10000. (A) Coomassie-blue stain of a gel that had received 7 µg ROS $\beta\gamma$, 5 µg bovine brain $\beta\gamma$, 9 µg human erythrocyte $\beta\gamma$ (RBC) and 5 µg human placental $\beta\gamma$. (B) Silver stain of a gel in which the protein loads were 10% of those in (A). (C) Silver stain of the same gal shown in (A).

The β and γ subunit composition of purified G proteins

The purification of G_s, G_{i2} and G_{i3}, essentially free from each other (Fig. 2) and from s single-cell-type human erythrocytes, offered an opportunity to explore whether the different G proteins might contain a distinctive complement of $\beta\gamma$ dimers. If so, this would mean that different $\beta\gamma$ dimers would be generated on activation of different G proteins, and hence that the dimers have a potential for mediation of G-protein-specific receptor signals. However, upon analysis of the β_{36}/β_{35} ratios of G_s, G_{i2} and G_{i3} purified from human erythrocytes, we found that each G protein contained the same relative proportions of β subunits (Fig. 3, lanes S, C, A and B). These data indicate that the ratio of affinities for β_{35} versus β_{36} containing $\beta\gamma$ dimers is the same for α_s, α_{i2} and α_{i3}.

Interaction of purified $\beta\gamma$ dimers with α subunits

Subunit makeup of the $\beta\gamma$ dimers used in the present study and complexity of the β-subunit makeup

Even though four cDNA species encoding highly similar β-subunit sequences have been cloned [13], to date only two of the gene products have been characterized in terms of their migration upon SDS/PAGE; β_1 which migrates as a polypeptide of M_r 36000 [38] and β_2 which migrates as a polypeptide of M_r 35000 [39, 40]. Since analysis by SDS/PAGE or urea gradient/SDS/PAGE (e.g. Fig. 4) has thus far revealed only β subunits that migrate with apparent M_r of 35000 (β_{35}) and 36000 (β_{36}), it follows that the translation products of β_3 and β_4 either co-migrate with those of β_1 and/or β_2, or that β_3 and β_4 are expressed only in selected tissues or cell types from which G proteins and/or $\beta\gamma$ dimers have not yet been purified. The $\beta\gamma$ dimers used in the present studies

contained widely differing ratios of β subunits with β_{35} and β_{36} behavior (Fig. 4). ROS $\beta\gamma$ dimers had no β_{35}, bovine brain $\beta\gamma$ had an approximate β_{36}/β_{35} ratio of 3:1, human erythrocyte $\beta\gamma$ dimers, which are obtained as a side product in the G-protein purification [21, 35], had about equal proportions of the two types of β subunits and human placental $\beta\gamma$ dimers [15] had an excess of β_{35} over β_{36} subunits of about 9–15:1.

Complexity of the γ subunit makeup

The four types of $\beta\gamma$ dimers differed not only in their β-subunit composition, but also in their γ-subunit composition, as analyzed by discontinuous-urea-gradient and polyacrylamide-gradient electrophoresis in the presence of SDS (Fig. 5). In each preparation, we found both a major band, denoted as γ_6, which in this system migrates slightly faster that a 6.2-kDa CNBr fragment of myoglobin, but co-migrates with it in a standard 10–15% polyacrylamide gradient SDS/PAGE, and a minor band, denoted as γ_8. The two forms of γ subunit differed according to the source of the $\beta\gamma$ dimer, both upon staining (Fig. 5C) and, as shown earlier, in immunological reactivity [15, 16] and the type of peptide maps they yield [41]. Thus, placental γ subunits stain differently from non-placental γ subunits, antibodies that cross-react with ROS γ subunits do not cross-react with bovine brain or human erythrocyte γ subunits [16], and antibodies that recognize placental γ subunits do not cross-react with either brain, liver or ROS γ subunits [16]. Likewise, peptide maps obtained from transducin γ_6 differ from those obtained from human erythrocyte G protein (or $\beta\gamma$) and bovine brain γ_6, which in turn do not differ significantly from each other [41]. Although this could suggest identity between human erythrocyte γ_6 and bovine brain γ_6, the peptide maps obtained from these bands were much more complex than expected from the amino acid sequence of a cloned bovine brain γ-subunit cDNA [42]. This raises the possibility that what appears as a poorly focused band may in fact represent a mixture of γ_6 peptides in both the erythrocyte and the brain proteins. The fact that up to four non-retinal γ subunits have been cloned [14], is consistent with this idea. The identity of the upper 'γ_8' peptides as true γ subunits has not yet been proven by cloning and/or immunological cross-reactivity, but is inferred from their apparent association with β subunits as seen by co-migration upon centrifugation through sucrose density gradients. Such co-migration has been shown for ROS γ_8 [43], as well as for human erythrocyte and bovine brain γ_8 (Codina, J. and Birnbaumer, L., unpublished results). However, this has not yet been shown for placental 'γ_8', so that its identity as a true γ subunit needs to remain tentative.

Thus, the four types of $\beta\gamma$ dimers used to test for specificity of their interaction with a common α subunit are quite heterogeneous, not only in the relative abundance of at least two types of β subunits, but also in the type of γ subunits associated with them, the latter being of at least three classes; retinal, placental and 'other.' A recombinant baculovirus α_{i3} was used to probe for differences in the α subunit association of these $\beta\gamma$ dimers.

Characteristics of purified recombinant baculovirus α_{i3} ($bv\alpha_{i3}$)

The details of the procedure for purification of this protein will be described elsewhere (Graf. R., Codina, J., Estes, M. K. and Birnbaumer L., unpublished results). The resulting protein was greater than 90% pure as determined by

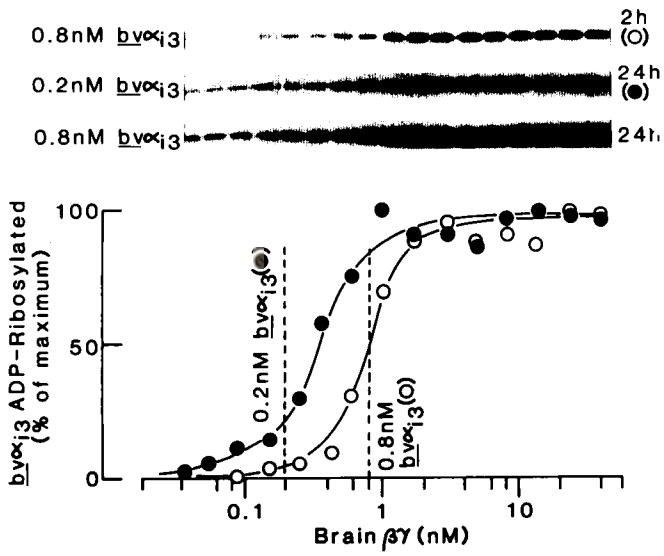

Fig. 6. Effect of increasing concentrations of bovine brain $\beta\gamma$ dimer on ADP-ribosylation of $bv\alpha_{i3}$ by PTX. Densitometric analysis of intensities of autoradiographic spots caused by [^{32}P]ADP-ribosylated $bv\alpha_{i3}$ of the reaction reagents separated by SDS/PAGE, as obtained by subjecting 0.2 nM (0.12 ng/15 µl assay; o) or 0.8 nM (0.48 ng/15 µl assay) $bv\alpha_{i3}$ to ADP-ribosylation by PTX in the presence of the indicated concentrations of bovine brain $\beta\gamma$ dimers. Top, photographs of autoradiographies of the [^{32}P]ADP-ribosylated $bv\alpha_{i3}$ obtained at the different $\beta\gamma$ dimer concentrations after 2 h (0.8 nM $bv\alpha_{i3}$) or 24 h (0.2 nm and 0.8 nM $bv\alpha_{i3}$) of exposure. Main portion of the figure, quantitative evaluation of autoradiographies. Data for the main panel were obtained by scanning the 2-h exposure of the reaction products obtained at 0.8 nM $bv\alpha_{i3}$ and the 24-h exposure of the reaction products obtained at 0.2 nM $bv\alpha_{i3}$. The figure shows only the regions of the gels in which proteins migrate with apparent molecular masse of 38000–43000 Da. No [^{32}P]ADP-ribosylation was detected upon ADP-ribosylating $\beta\gamma$ dimers alone (not shown). ADP-ribosylations were for 36 h at 4°C in a final volume of 15 µl under conditions described in Materials and Methods.

Coomassie-blue staining, bound 0.7 ± 0.2 mol [^{35}S]GTP[γS]/mol protein, assuming a M_r of 40000 (not shown), and contained only one major contaminant (1% – 5% of the total, depending on the preparation) that migrated on SDS/PAGE close to the major $bv\alpha_{i3}$ band (Fig. 1). Upon immunoblotting, this band, as well as the major $bv\alpha_{i3}$ band, cross-reacted with anti-peptide IgG specific for α_{i3} (not shown). Among the possibilities are that the lower band is either a proteolytic fragment of the translated $bv\alpha_{i3}$, the result of internal initiation (e.g. Met18) or early termination of translation of the $bv\alpha_{i3}$ mRNA, the product of an alternatively spliced $bv\alpha_{i3}$ mRNA, or a correctly translated α_{i3} with different post-translational modifications. When subjected to ADP-ribosylation by PTX, alone or in the presence of a $\beta\gamma$ dimer, the upper but not the lower band is labeled (Fig. 1), indicating that the band of interest with α-subunit properties is indeed the upper and not the lower.

ADP-ribosylation of $bv\alpha_{i3}$ depends on the concentration of $\beta\gamma$ dimers

Under our assay conditions (see Material and Methods), we observed that ADP-ribosylation of $bv\alpha_{i3}$ is greatly accelerated by $\beta\gamma$ dimers and that, over a limited range of concentrations, the proportion of α-subunit ADP-ribosylated is proportional to the amount of $\beta\gamma$ added. This is illustrated for two concentrations of $bv\alpha_{i3}$ in Fig. 6. On the basis of results

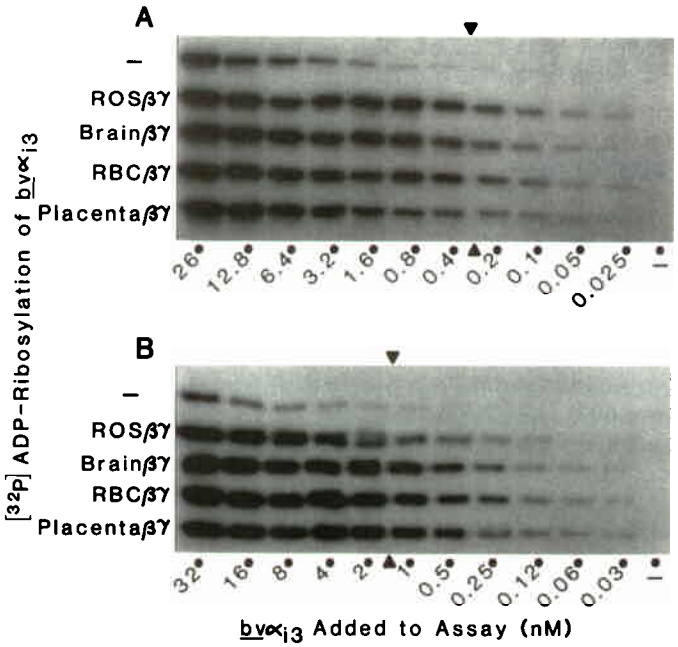

Fig. 7. Comparison of the ability of four types of $\beta\gamma$ dimers to promote ADP-ribosylation of a G-protein α subunit ($bv\alpha_{i3}$). $\beta\gamma$ dimers were mixed with increasing concentrations of $bv\alpha_{i3}$, PTX (10 μg/ml) and ADP-ribosylation reagents and incubated at 4°C for 36 h. The total reaction mixture (10 μl for A and 15 μl for B) was diluted in NAD and SDS-containing Laemmli sample buffer [24] and subjected to SDS/PAGE, stained with Coomassie blue and autoradiographed. The regions of the autoradiographies corresponding to [^{32}P]ADP-ribosylated $bv\alpha_{i3}$, as obtained without $\beta\gamma$ addition (control), and with addition of either bovine brain, human erythrocyte (RBC) or human placental $\beta\gamma$ dimers were superimposed, slightly offset so as to allow comparison, and photographed. (A) ADP-ribosylation of the indicated concentrations of α subunits in the absence (top row) or the presence of 0.3 nM of the four types of $\beta\gamma$ dimer (next four rows); (B) ADP-ribosylation of the indicated concentrations of α subunit in the absence (top row) or presence of 1.6 nM of four $\beta\gamma$ dimers (next four rows). The $\beta\gamma$ dimer preparations used were those analyzed in Figs 4 and 5.

such as presented on Fig. 6, we concluded that ADP-ribosylation by PTX can be used as a measure of the ability of $\beta\gamma$ dimers to interact with the α subunit.

On a quantitative level, under the assay conditions used, which included a 36-h incubation in the cold room and the presence of a 10-fold molar excess of $\beta\gamma$ dimer, we obtained a maximal incorporation of ADP-ribose of 0.7 mol/mol $bv\alpha_{i3}$ protein, equal to the GTP[γS]-binding activity of the preparation. We obtained only 0.2–0.3 mol ADP-ribose incorporated/mol protein substrate when incubations were performed at 32°C, regardless of the time of incubation.

Comparison of the ability of the four preparations of $\beta\gamma$ dimers to promote ADP-ribosylation of $bv\alpha_{i3}$ by PTX

Fig. 7 presents typical results obtained in two of several experiments, in which fixed concentrations of $\beta\gamma$ dimers (0.3 nM and 1.6 nM in this case) were mixed with increasing concentrations of $bv\alpha_{i3}$, then subjected to ADP-ribosylation by 10 μg/ml PTX in the presence of 300 nM [^{32}P]NAD^+. Several aspects of the results are worth mentioning. First, none of the $\beta\gamma$-dimer preparations were significantly contaminated by PTX substrates, as seen in the last lanes with samples from ADP-ribosylation reactions without $bv\alpha_{i3}$. Second, association with $\beta\gamma$ does not seem to be an absolute requirement for ADP-ribosylation of the $bv\alpha_{i3}$ by PTX. ADP-ribosylation in the absence of added $\beta\gamma$ persisted after centrifugation through a sucrose density gradient and collection of the α subunit from the 2S region of the gradient, indicating that the ADP-ribosylation is not likely to be due to contaminating baculovirus $\beta\gamma$. We have not yet completed all the studies, but it would seem that the effect of $\beta\gamma$ dimers is to accelerate the rate at which α subunits are ADP-ribosylated by a given concentration of PTX. Third, the enhancement of ADP-ribosylation by $\beta\gamma$ dimers is between 16–32-fold, as deduced from dilution ratios of minimally labeled samples. Finally, as can be seen from the comparison of the superimposed autoradiograph, the four types of $\beta\gamma$ dimers were, within a factor of 2–3, about equipotent in stimulating the ADP-ribosylation of the α subunit by PTX. For these experiments, the concentrations of $\beta\gamma$ dimers were estimated on the basis of the Coomassie-blue-staining intensity of their combined β subunits, as seen in SDS/PAGE using, as standard, the staining intensity of varying amounts of bovine serum albumin electrophoresed on the same gel slabs. Staining intensities were determined with the aid of a Helena Laboratories Gel Scanner (Beaumont, TX).

ROS $\beta\gamma$ dimers differ from $\beta\gamma$ dimers derived from placental or brain membranes when assayed in terms of their interaction with α_s

The results related thus far were not what we had expected on the basis of our previous studies that compared either the efficacy with which bovine brain and bovine ROS $\beta\gamma$ dimers inhibited adenylyl cyclase activity, as seen upon reconstitution of purified G_s and a resolved G-protein-free preparation of adenylyl cyclase in phospholipid vesicles [44], or the efficacy with which these same $\beta\gamma$ dimers inhibit G_k-activated K^+-channel activity in inside-out membrane patches [45]. In both of these studies, half maximal effects required at least 10-fold higher concentrations of the retinal rod cell $\beta\gamma$ dimers, which are hydrophilic, than of the bovine brain $\beta\gamma$ dimers, which are hydrophobic and required detergent to remain in solution prior to their use. A similar difference in efficacy was reported also by Casey et al. [45] who tested the ability of bovine brain and ROS $\beta\gamma$ dimers to deactivate fluoride activated G_s. One explanation as to why the relative potencies with which different $\beta\gamma$ dimers act in the different assays could be a uniqueness of the rod cell $\beta\gamma$ dimer, another could be that both G_s and the particular G protein that fulfills the role of G_k in guinea pig atrial membranes are more sensitive to molecular differences than recombinant α_{i3}.

We tested whether the retinal $\beta\gamma$ dimers might be unique by comparing these dimers to $\beta\gamma$ dimers from bovine brain and human placenta, using two assays that may be more sensitive to differences in $\beta\gamma$ composition; (a) the deactivation of fluoride-activated G_s purified from human erythrocyte membranes [46, 47] and (b) inhibition of adenylyl cyclase activity in intact membranes [18]. As shown in Fig. 8 and Table 1, when $\beta\gamma$ function is tested in these ways, the ROS $\beta\gamma$ is clearly less potent or efficacious in interacting with α_s, than both the bovine brain and the human placental $\beta\gamma$ dimers. The human placental and bovine brain $\beta\gamma$ dimers, in contrast, were indistinguishable in both α_s interaction assays.

The experiment of Table 1 shows that the differences in the effectiveness of $\beta\gamma$ dimers to interact with α_s, seen with purified protein in detergent solution, also hold true when $\beta\gamma$ dimers are tested by addition to intact membranes. The finding

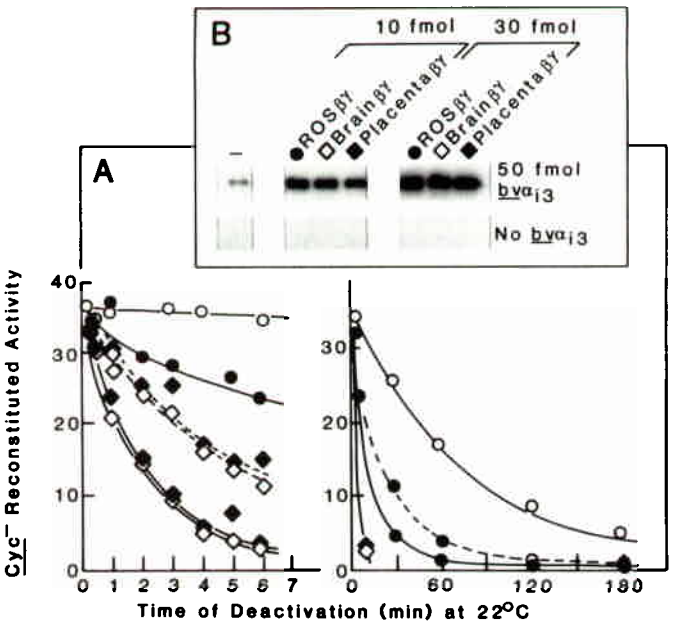

Fig. 8. Comparison of the ability of three $\beta\gamma$ dimers to accelerate the deactivation of G_s that had been activated in the with NaF and AlC_3. Activation: 5 nM human erythrocyte G_s was incubated for 20 min at 32 °C in the presence of 6 mM $MgCl_2$, 10 mM NaF, 10 μM $AlCl_3$, 1% bovine serum albumin, 20 mM 2-mercaptoethanol, 1 mM EDTA, 0.1% Lubrol PX, 10 mM Hepes (sodium salt), pH 8.0, and 15% ethylene glycol. Deactivation: 1 μl [50 fmol/tube (—) of activated G_s was mixed with 10 μl 1% bovine serum albumin, 0.1% Lubrol PX, 20 mM 2-mercaptoethanol, 5 mM EDTA and 50 mM Tris/HCl, pH 7.6, containing a 5-nM (50 fmol/tube) or 15-nM [150 fmol/tube (- - -) concentration of the $\beta\gamma$ dimer and incubated at room temperature for the times shown. (A) Assay of G_s activity. 90 μl reagents for determination of reconstitution and measurement of adenylyl cyclase *cyc*⁻ membrane adenylyl cyclase [10 μl 125 mM Tris/HCl, pH 7.6, 10 mM EDTA, 100 mM $MgCl_2$ 10 mM [^{3}H]cAMP (12.800 cpm)]; 10 μl nucleoside triphosphate regenerating system formed of 200 mM creatine phosphate, 20 mg/ml creatine phosphokinase and 2 mg/ml adenylate kinase; 20 μl 0.5 mM [α-^{32}]ATP (23×10^6 cpm), and 50 μl 0.2% bovine serum albumin containing 10 μg *cyc*⁻ membrane protein) were added to the 11 μl of the mixture in which G_s had been allowed to deactivate and incubated for 20 min at 30 °C. The reactions were terminated by addition of 100 μl 40 mM ATP, 10 mM cAMP and 1% SDS and the [^{32}P]cAMP formed was isolated by double-column chromatography ([4, 55] and quantified by liquid scintillation counting). (B) Assay of $\beta\gamma$ dimer added to the deactivation reaction. 2.5 μl 20 mM 2-mercaptoethanol, 1 mM EDTA and 30% ethylene glycol either with or without 25 nM bvα$_{i3}$, were mixed with 2.5 μl of the same dilutions of $\beta\gamma$ dimers as used for the deactivation reaction and 10 μl labeling reagents (2.5 μl 0.6 μg/ml activated PTX solution, 2.5 μl 6 μM [^{32}P]NAD^+ (4000000 cpm), 5 μl 30 mM thymidine, 300 μM GDP[βS], 3 mM adenyl-5'yl imidodiphosphate, and incubated at 4 °C for 36 h. The complete reaction mixtures were then mixed with 15 μl twice-concentrated Laemmli's sample buffer containing, in addition, 10 mM NAD^+, and analyzed by SDS/PAGE and autoradiography of labeled bands. Note that equivalent amounts of $\beta\gamma$ dimers had been added to the deactivation assay. Time axis of (A), time of incubation at room temperature for deactivation. Cyc⁻ reconstituted activity in pmol · min^{-1} · mg^{-1}. ◆, placental $\beta\gamma$; ◇, brain $\beta\gamma$; ●, ROS $\beta\gamma$; ○, no addition (5 fmol G_s^* tube).

that addition of a stimulatory hormone decreases the effect of $\beta\gamma$ dimers to inhibit adenylyl cyclase is in agreement with a similar finding by Hildebrandt and Kohnken [48]. Experiments with GH_4C_1 cell homogenates to be published elsewhere (Codina J. and Birnbaumer, L., unpublished results), showed that the phenomenon by which stimulation of adenylyl cyclase inhibits the inhibition by $\beta\gamma$ dimers is not restricted to the S49 adenylyl cyclase system.

DISCUSSION

The present finding that, as isolated from a single membrane, different G proteins exhibit the same heterogeneity in $\beta\gamma$ composition (Fig. 3), suggests that $\beta\gamma$ dimers constitute a heterogenous pool of which the members can be shared among all G proteins in a non-discriminating manner. It follows, therefore, that α_s, α_{i2} and α_{i3} do not exhibit noticeable differences in affinity for one over the other of the two (or more) $\beta\gamma$ dimers expressed in these cells. This is not to say, of course, that the affinities of the different α subunits for $\beta\gamma$ dimers may not vary.

In spite of this observed lack of selectivity in the association of an α subunit for any given $\beta\gamma$ dimer, the results do not allow us to extrapolate to the situation in the intact cell. Thus, while the purification procedure used by us [21, 35] and legend to Fig. 2) rigorously avoids fluoride ions and any guanine nucleotide, we cannot assert that mere extraction of G proteins from the erythrocyte membrane (with cholate and Mg^{2+} in the presence of 2-mercaptoethanol) may have randomized subunit composition. If this is what happened, then the composition of the purified proteins is in fact the result of an *in-vitro* subunit interaction assay, rather than an assay of *in-vivo* subunit composition. This *in-vitro* subunit interaction assay showed total lack of selectivity of one α for any one of the $\beta\gamma$ subunits.

The molecular basis for the relative lack of retinal $\beta\gamma$ dimers to promote deactivation of G_s in solution (Fig. 8) or inhibit GTP-mediated activation of G_s in membranes (Table 1), is not known at this time. We do not think it is due to solvent and/or detergent effects. Although ROS $\beta\gamma$ subunits are purified without detergents, they were diluted 2000-fold (from 35 μM in the storage buffer to 15 nM in the G_s-deactivation assay) with the same Lubrol and 2-mercaptoethanol-containing buffer in which placental and brain $\beta\gamma$ dimers are kept and diluted. We feel that the unique behavior of ROS $\beta\gamma$ subunits is likely to be due to their unique γ subunit(s) [41] and/or to different post-translational modifications of the β_{36} and/or γ subunits. Thus, even though the primary amino acid sequences of the retinal and brain β_{36} are the same by peptide mapping [41, 49], immunological reactivity towards a peptide-directed antibody [38] and cloning [50, 51], their amino termini are blocked (Codina, J., Cook, R. and Birnbaumer, L., unpublished results). This indicates the existence of at least one post-translational modification, the chemical nature of which is unknown and which could vary with the cell type. In addition, it has now been well established that the γ subunit expressed in retinal rod cells, known as γ_T or γ_1, not only differs by as much as 30% in its primary amino acid sequence [14], but also in the nature of its carboxy-terminal polyisoprenylation. γ_T is farnesylated, γ subunits elsewhere are geranylgeranylated [52]. It is thus conceivable that, by differently modifying γ and β subunits, rod cells create a separate pool of $\beta\gamma$ dimers, designed to interact primarily with the α subunit of transducin and thus participate in the signal transduction of light, without interfering with other G protein mediated signal transductions, e.g. adenylyl cyclase and various phospholipases which are known to exist in these same cells and operate in response to humoral signals independently of the lighting condition. In this sense, the functional difference uncovered

Table 1. Comparison of inhibition of wild-type S49 cell adenylyl cyclase activities by bovine brain, human placental and ROS $\gamma\beta$ dimers. 25 μg protein of S49 cell membranes [35000 × *g* pellet prepared as described by Ross et al. [53] with the exception that $MgCl_2$ was omitted from the buffers and resuspended in 1 mM EDTA and 10 mM Hepes (sodium salt), pH 8.0)] were mixed in a final volume of 40 μl with 5 μl 10 mM EDTA, 20 mM $MgCl_2$, 10 mM [^{3}H]cAMP (12750 cpm) and 125 mM Hepes (sodium salt), pH 8.0, 5 μl 20 mM creatine phosphate, 20 mg/ml creatine phosphokinase, 2 mg/ml adenylate kinase, 5 μl 600 mM NaCl, 5 μl 1 mM ATP and 100 μM GTP and 10 μl of 100 mM NaCl, 1 mM EDTA, 10 mM Hepes (sodium salt), pH 8.0, 0.02% Lubrol PX, 2 mM 2-mercaptoethanol and 3% ethylene glycol either with or without the indicated $\beta\gamma$ dimers. These mixtures were incubated on ice for 30 min and then at 32 °C for 15 min. Adenylyl cyclase activity was then measured for 15 min at 32 °C after addition of 10 μl 0.1% bovine serum albumin containing 18.7×10^6 cpm of [α-^{32}P]-ATP (450 Ci/mmol) without or with 50 μM (−)-isoproterenol. The reactions were stopped and [^{32}P]cAMP was then determined as described previously [54, 55]. Incubations were carried out in triplicates and values are means ± S. D. Similar results were obtained in a total of three experiments of this type. All activities in the presence of $\beta\gamma$ dimers were lower than control at $P < 0.05$. All effects of $\beta\gamma$ dimers were decreased by addition of isoproterenol at $P < 0.05$. The control values are given by the inhibition of the effect of the $\beta\gamma$ dimer in the presence of isoproterenol.

Addition	Adenylyl Cyclase Activity in the presence of		Inhibition of Activity		Inhibition of the effect of $\beta\gamma$ by isoproterenol
	−	10 μM isoproterenol	−	10 μM isoproterenol	
	pmol · min^{-1} · mg protein^{-1}		%		
No addition	3.8 ± 0.1	78 ± 3	−	−	−
Brain $\beta\gamma$					
0.66 pmol	2.3 ± 0.1	68 ± 5	40 ± 2	13 ± 8	68 ± 19
2.00 pmol	1.8 ± 0.1	47 ± 3	53 ± 4	40 ± 4	25 ± 7
0.66 pmol	2.2 ± 0.1	68 ± 4	43 ± 3	13 ± 8	70 ± 13
2.00 pmol	1.7 ± 0.1	47 ± 4	56 ± 5	40 ± 4	29 ± 9
ROS $\beta\gamma$					
0.66 pmol	3.2 ± 0.1	76 ± 3	16 ± 3	3 ± 4[a]	89 ± 25
2.00 pmol	2.6 ± 0.2	66 ± 1	32 ± 6	15 ± 4	53 ± 15

[a] Activity in the presence of $\beta\gamma$ dimers was the same as that of the control.

for ROS $\beta\gamma$ may be unique and have no general physiologic relevance.

We have thus analysed the properties of $\beta\gamma$ dimers, both in terms of structure as seen on polyacrylamide-gel electrophoresis and differential staining properties, and in terms of functional interactions with purified and native membrane-bound α subunits. We conclude that the analysis of G proteins of one cell type, the erythrocyte, does not support the idea that one α subunit interacts preferentially with one type of $\beta\gamma$ molecule over another. With the exception of the ROS $\beta\gamma$, this conclusion is supported by the findings that $\beta\gamma$ subunits are about equipotent in promoting ADP-ribosylation of a recombinant α subunit and that brain and placental $\beta\gamma$ subunits are equipotent in promoting deactivation of fluoride-activated G_s, or in interfering with α_s-dependent basal adenylyl cyclase activity.

Taken together, these data are not conducive to thoughts that would ascribe specific signalling roles to one or the other of the $\beta\gamma$ dimers. However, the subunit composition of $\beta\gamma$ dimers is very complex, as revealed in the proteins isolated from different tissues, and, although an *in-vitro* assay such as G_s deactivation may not be of immediate physiologic relevance, it shows that it is possible to measure a functional differences for at least one of the $\beta\gamma$ dimers, the ROS $\beta\gamma$. This obliges one to consider the possibility that the $\beta\gamma$ composition of a given G protein could vary selectively under non-steady-state conditions and that this could confer differing temporal $\beta\gamma$-dimer patterns to the activation of a G protein. To resolve this issue, it will be necessary to determine not only affinities of individual $\beta\gamma$ dimers for G-protein α subunits, but also for the other coupling partners, the receptors and the effectors.

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REFERENCES

1. Birnbaumer, L., Codina, J., Mattera, R., Cerione, R. A., Hildebrandt, J. D., Rojas, F. J., Caron, M. C., Lefkowitz, R. J. & Iyengar, R. (1985) Regulation of Hormone Receptors by Guanine Nucleotide Binding N Proteins, *Rec. Prog. Horm. Res. 41*, 41–94.
2. Gilman, A. G. (1987) G Proteins: Transducers of Receptor-Generated Signals, *Annu. Rev. Biochem. 56*, 615–649.
3. Neer, E. J. & Clapham, D. E. (1988) Roles of G Protein Subunits in Transmembrane Signalling, *Nature 333*, 129–134.
4. Federman, A. D., Conklin, B. R., Schrader, K. A., Reed, R. R. & Bourne, H. R. (1992) Hormonal Stimulation of Adenylyl Cyclase Through G_i-protein $\beta\gamma$ Subunits, *Nature 356*, 159–161.5. Birnbaumer, L. (1990) G Proteins in Signal Transduction, *Annu. Rev. Pharmacol. Toxicol. 30*, 675–705.
6. Birnbaumer, L. (1987) Which G Protein Subunits are the Active Mediators in Signal Transduction, *Trends Pharmacol. Sci. 8*, 209–211.
7. Bourne, H. R. (1987) 'Wrong' Subunit Regulates Cardiac Potassium Channels, *Nature 325*, 296–297.
8. Bourne, H. R. (1989) Who Carries What Message?, *Nature 337*, 504–505.
9. Dietzel, C. & Kurjan, J. (1987), The Yeast SCG1 Gene: A Gα-like Protein Implicated in the a- and α-Factor Response Pathway, *Cell 50*,1001–1010.
10. Miyajima, I., Nakafuku, M., Nakayama, N., Brenner, C., Miyajima, A., Kalbuchi, K., Arai, K.-I., Kaziro, Y. & Matsumoto, K. (1987) GPA1, A Haploid-Specific Essential Gene, Encodes a Yeast Homolog of Mammalian G Protein Which May be Involved in Mating Factor Signal Transduction, *Cell 50*, 1011–1019.
11. Whiteway, M. Hougan, L., Dignard, D., Thomas, D. Y., Bell, L., Saari, G. C., Grant, F. J., O'Hara, P. & MacKay, V. L. (1989) The STE4 and STE18 Genes of Yeast Encode Potential β and

γ Subunits of the Mating Factor Receptor-Coupled G Protein, *Cell 56*, 467–477.
12. Tang, W. J. & Gilman, A. G. (1991) Type Specific Regulation of Adenylyl Cyclase by G Protien $\beta\gamma$ Subunits, *Science 254*, 1500–1503.
13. Simon, M. I., Strathmann, M. P. & Gautam, N. (1991) Diversity of G proteins in Signal Transduction, *Science 252*, 802–808.
14. Fisher J. K. & Aronson, N. N. (1992) Characterization of the cDNA and Genomic Sequence of a G Protein γ Subunit (γ_5), *Mol. Cell. Biol. 12*, 1585–1591.
15. Gierschik, P., Codina, J., Simons, C., Birnbaumer, L. & Spiegel, A. (1985) Antisera Against a Guanine Binding Protein from Retina Cross-react with the beta Subunit of the Adenylyl Cyclase Associated Guanine Nucleotide Binding Proteins, N_s and N_i, *Proc. Natl Acad. Sci. USA 82*, 721–727.
16. Evans, T., Fawzi, A., Fraser, E. D., Brown, M. L. & Northup, J. K. (1987) Purification of a $Beta_{35}$ Form of the Beta-Gamma Complex Common to G-Proteins from Human Placental Membranes, *J. Biol. Chem. 262*, 176–181.
17. Northup, J. K., Smigel, M. D., Sternweis, P. C. & Gilman, A. G. (1983) The Subunits of the Stimulatory Regulatory Component of Adenylate Cyclase. Resolution of the Activated 45000-Dalton (alpha) Subunit, *J. Biol. Chem. 258*, 11369–11376.
18. Katada, T., Bokoch, G. M., Northup, J. K., Ui, M. & Gilman, A. G. (1984) The Inhibitory Guanine Nucleotide-binding Regulatory Component of Adenylate Cyclase. Properties and Function of the Purified Protein, *J. Biol. Chem. 259*, 3568–3577.
19. Kanaho, Y., Tsai, S.-C., Adamik, R., Hewlett, E. L., Moss, J. & Vaughan, M. (1984) Rhodopsin-enhanced GTPase Activity of the Inhibitory GTP-binding Protein of Adenylate Cyclase, *J. Biol. Chem. 259*, 7378–7381.
20. Tsai, S.-C., Adamik, R., Kanaho, Y., Hewlett, E. L. & Moss, J. (1984) Effects of Guanyl Nucleotides and Rhodopsin on ADP-ribosylation of the Inhibitory GTP-binding Component of Adenylate Cyclase by Pertussis Toxin, *J. Biol. Chem. 259*, 15320–15323.
21. Codina, J., Hildebrandt, J. D., Sekura, R. D., Birnbaumer, M., Bryan, J., Manclark, C. R., Iyengar, R. & Birnbaumer, L. (1984) N_s and N_i, the Stimulatory and Inhibitory Regulatory Components of Adenylyl Cyclases. Purification of the Human Erythrocyte Proteins Without the Use of Activating Regulatory Ligands, *J. Biol. Chem. 259*, 5871–5886.
22. Walseth, T. F. & Johnson, R. A. (1979) The Enzymatic Preparation of [α-^{32}P] Nucleoside Triphosphate, Cyclic [^{32}P]AMP and cyclic [^{32}P] GMP, *Biochem. Biophys. Acta 562*, 11–31.
23. Cassel, D. & Pfeuffer, T. (1978) Mechanism of Cholera Toxin Action: Covalent Modification of the Guanyl Nucleotide-Binding Protein of the Adenylate Cyclase System, *Proc. Natl Acad. Sci. USA 75*, 2669–2673.
24. Laemmli, U. (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4, *Nature 227*, 680–685.
25. Scherer, N. M., Toro, M.-J., Entman, M. L. & Birnbaumer, L. (1987) G Protein Distribution in Canine Cardiac Sarcoplasmic Reticulum and Sarcolemma. Comparison to Rabbit Skeletal Membranes and Brain and Erythrocyte G Proteins, *Arch. Biochem. Biophys. 259*, 431–440.
26. Codina, J., Grenet, D., Chang, K.-J. & Birnbaumer, L. (1991) Urea Gradient/SDS/PAGE: A Useful Tool in the Investigation of Signal Transducing G Proteins, *J. Receptor Res. 11*, 587–601.
27. Hildebrandt, J. D., Codina, J., Risinger, R. & Birnbaumer, L. (1984) Identification of a γ Subunit Associated with the Adenylyl Cyclase Regulatory Proteins N_s and N_i, *J. Biol. Chem. 259*, 2039–2042.
28. Sammons, D. W., Adams, L. D., Nishizawa, E. E. (1981) Ultrasensitive Silver-based Color Staining of Polypeptides in Polyacrylamide Gels, *Electrophoresis 2*, 135–141.
29. Green, N., Alexander, H., Olson, A., Alexander, S., Shinnick, T. M., Suttliffe, J. G. & Lerner, R. A. (1982) Immunogenic Structure of the Influenza Virus Hemagglutinin, *Cell 28*, 477–487.
30. Didsbury, J. R., & Snyderman, R. (1987) Molecular Cloning of a New Human G Protein. Evidence for Two $G_1\alpha$-like Protein Families, *FEBS Lett. 219*, 259–263.
31. Suki, W., Abramowitz, J., Mattera, R., Codina, J. & Birnbaumer, L. (1987) The Human Genome Encodes at Least Three Nor Allelic G Proteins with $alpha_i$-type Subunits, *FEBS Lett. 220*, 187–192.
32. Codina, J., Olate, J., Abramowitz, J., Mattera, R., Cook, R. G. & Birnbaumer, L. (1988) '*Alpha*$_i$–3 cDNA Encodes he alpha Subunit of G_k, the Stimulatory G Protein of Receptor-regulated K^+ Channels, *J. Biol. Chem. 263*, 6746–6750.
33. Smith, G. E., Ju, G., Ericson, B. L., Moschera, J., Jahm, H. W., Chizzonite, R. & Summars, M. D. (1985) Modification and Secretion of Human Interleukin 2 Produced in Insect Cells by a Baculovirus Expression Vector, *Proc. Natl Acad. Sci. USA 82*, 8404–8408.
34. Luckov, V. A. & Summers, M. D. (1988) Trends in the Development of Baculo-virus Expression Vectors, *Bio/Technol. 6*, 47–55.
35. Codina, J., Rosenthal, W., Hildebrandt, J. D., Sekura, R. D. & Birnbaumer, L. (1984) Updated Protocols and Comments on the Purification Without Use of Activating Ligands of the Coupling Proteins N_s and N_i of the Hormone Sensitive Adenylyl Cyclase, *J. Receptor Res. 4*, 411–422.
36. Codina, J., Yatani, A., Grenet, D., Brown, A. M. & Birnbaumer, L. (1987) The alpha Subunit of the GTP Binding Protein G_k Opens Atrial Potassium Channels, *Science 236*, 442–445.
37. Ho, Y.-K. & Fung, B. K.-K. (1984) Characterization of Transducin from Bovine Retinal Rod Outer Segments. The Role of Sulfhydryl Groups, *J. Biol. Chem. 259*, 6694–6699.
38. Mumby, S. M., Kahn, R. A., Manning, D. R. & Gilman, A. G. (1986) Antisera of Designed Specificity for Subunits of Guanine Nucleotide-binding Regulatory Proteins, *Proc. Natl Acad. Sci. USA 83*, 265–269.
39. Gao, B., Mumby, S. & Gilman, A. G. (1987) The G Protein β_2 Complementary DNA Encodes the β_{35} Subunit, *J. Biol. Chem. 262*, 17254–17257.
40. Amatruda, T. T. III, Gautam, N., Fong, H, K. W., Northup, J. K. & Simon, M. I. (1988) The 35- and 36-kDa β Subunits of GTP-binding Regulatory Proteins are Products of Separate Genes, *J. Biol. Chem. 263*, 5008–5011.
41. Hildebrandt, J. D., Codina, J., Rosenthal, W., Birnbaumer, L., Neer, E. J., Yamazaki, A. & Bitensky, M. W. (1985) Characterization by Two-Dimensional Peptide Mapping of the Subunit Composition of the Regulatory N_s and N_i Proteins of Adenylyl Cyclase, and Transducin, the Guanine Nucleotide Binding Protein of Retinal Rod Outer Segments, *J. Biol. Chem. 260*, 14867–14872.
42. Gautam, N., Baetscher, M., Aebersold, R., Simon, M. I. (1989) A G Protein Gamma Subunit Shares Homology with rase Proteins, *Science 244*, 971–974.
43. Fukada, Y., Ohguro, H., Saito, T., Yoshizawa, T. & Akino, T. (1989) $\beta\gamma$-Subunits of Bovine Transducin Composed of Two Components with Distinctive γ-Subunits, *J. Biol. Chem. 264*, 5937–5943.
44. Cerione, R. A., Gierschik, P., Staniszewski, C., Benovic, J. L., Codina, J., Somers, R., Birnbaumer, L., Spiegel, A. M., Lefkowitz, R. J. & Caron, M. G. (1987) Functional Differences in the beta-gamma Complexes of Transducin and the Inhibitory Guanine Nucleotide Regulatory Protein, *Biochemistry 26*, 1485–1491.
45. Okabe, K., Yatani, A., Evans, T., Ho Y.-K., Codina J., Birnbaumer, L. & Brown, A. M. (1990) $\beta\gamma$ Dimers of G Proteins Inhibit Muscarinic K^+ Channels in Heart, *J. Biol. Chem. 265*, 12854–12858.
46. Casey, P. J., Graziano, M. P. & Gilman, A. G. (1989) G Protein $\beta\gamma$ Subunits from Bovine Brain and Retina: Equivalent Catalytic Suppoprt of ADP-ribosylation of α Subunits by Pertussis Toxin but Differential Interactions with $G_s\alpha$, *Biochemistry 28*, 611–616.
47. Northup, J. K., Sternweis, P. C. & Gilman, A. G. (1983) The Subunits of the Stimulatory Regulatory Component of

Adenylate Cyclase. Resolution, Activity and Properties of the 35000 Dalton (beta) Subunit, *J. Biol. Chem. 258*, 11361–11368.

48. Hildebrandt, J. D. & Kohnken, R. E. (1990) Hormone Inhibition of Adenylyl Cyclase, *J. Biol. Chem. 265*, 9825–9830.
49. Manning, D. R. & Gilman, A. G. (1983) The Regulatory Components of Adenylate Cyclase and Transducin. A Family of Structurally Homologous Guanine Nucleotide-Binding Proteins, *J. Biol. Chem. 258*, 7059–7063.
50. Sugimoto, K., Nukada, T., Tanabe, T., Takahashi, H., Noda, M., Minamino, N., Kangawa, K., Matsuo, H., Hirose, T., Inayama, S. & Numa, S. (1985) Primary Structure of the Beta-subunit of Bovine Transducin Deduced from the cDNA Sequence, *FEBS Lett. 191*, 235–240.
51. Codina, J., Stengel, D., Woo, S. L. C. & Birnbaumer, L. (1986) Beta Subunits of the Human Liver G_s/G_i Signal Transducing Proteins and Those of Bovine Retinal Rod Cell Transducin are Identical, *FEBS Lett. 207*, 187–192.
52. Sanford, J., Codina, J., & Birnbaumer, L. (1991) γ-Subunits of G Proteins, But Not Their α- or β-Subunits, Are Polysioprenylated. Studies on Post-translational Modifications Using *in vitro* Translation with Rabbit Reticulocyte Lysates, *J. Biol. Chem. 266*, 9570–9579.
53. Ross E. M., Maguire M. E., Sturgill T. W., Biltonen R. L. & Gilman A. G. (1977) Relationship Between the Beta-adrenergic Receptor and Adenylate Cyclase. Studies of Ligand Binding and Enzyme Activity in Purified Membranes of S49 Lymphoma Cells, *J. Biol. Chem. 252*, 5761–5775.
54. Salomon, Y., Londos, C. & Rodbell, M. (1974) A Highly Sensitive Adenylate Cyclase Assay, *Anal. Biochem. 58*, 541–548.
55. Bockaert, J., Hunzicker-Dunn, M. & Birnbaumer, L. (1976) Hormone-stimulated Desensitization of Hormone-dependent Adenylyl Cyclase. Dual Action of Luteinizing Hormone on Pig Graafian Follicle Membranes, *J. Biol. Chem. 251*, 2653–2663.